PRODUCTION OF RECOMBINANT SHEEP PRION PROTEIN (RecShPrP^{C})

AND ITS DETECTION IN MUSCLE FOOD USING WESTERN BLOTTING

AND IMMUNO-PCR

By

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FORMAT OF THESIS

This thesis is presented in the format of Journal of Animal Science, as outlined by the Oklahoma State University graduate college style manual. The use of this format allows independent chapters to be suitably prepared for submission for scientific journals.

CHAPTER I

INTRODUCTION

In the year 1993, the beef industry was struggling with the implications of the Jack-in-the-Box *Escherichia coli* O157: H7 tragedy and for the next 10 years, food scientists spent their time finding ways of reducing or eliminating *E. coli*O157: H7. The disaster compelled many packers and further processors of beef to go out of business and the industry incurred more than $2.7 billion in loses.

Today we hardly see any case of *E. coli*O157: H7 contamination or any recall related to it making headlines. However, the lingering problem in the beef industry today is the rise of a new fatal and transmissible disease among cattle called Bovine Spongiform Encephalopathy (BSE). The first confirmed case of BSE or mad cow disease in United States was reported in December of 2003 in a Washington state dairy cow. This discovery halted billions of dollars worth of beef exports and caused severe economic and political impacts across the nation.

Bovine spongiform encephalopathy belongs to a group of diseases called prion diseases or transmissible spongiform encephalopathies (TSE) which are a set of fatal neurodegenerative disorders caused by modification of prion protein (PrP), a constituent of all the mammalian cells to an abnormal isoform designated PrP^{Sc} (Prusiner, 1991). The other prion diseases are scrapie in sheep and goats, kuru, Creutzfeldt-Jakob disease (CJD), Gerstmann-Straussler syndrome (GSS), and fatal familial insomnia (FFI) in human beings and encephalopathies in mink, cats, mule deer, and elk (Masters et al., 1981; Medori et al., 1992; Gajdusek, 1997). Prion diseases have generated a lot of interest in the scientific community not only because of their unique biochemical

characteristics but also because of their potential threat and impacts on the human health (Harris, 1999). Over the past decade, more than 140 patients have died because of the new variant form of Creutzfeldt-Jakob disease (vCJD) caused by the consumption of beef contaminated with BSE material. The contamination of the human food supply occurs when the BSE material from mechanically recovered meat (MRM) containing the central nervous system material of the cattle is mixed with processed meat. Though most cases of BSE that occurred in European countries were indigenous in nature, these diseases can occur in any part of the world where BSE exists (Brown et al., 2003). The US government, as elsewhere, has taken necessary steps not only to prevent the contamination of the human food supply with specified risk materials (SRM) coming from the by-products of a slaughtered cattle but also have acted to break the cycle by which the disease spreads from one animal to another(Brown et al., 2003). However, the application of these regulations and strategies needs continuous monitoring to ensure compliance (Brown et al., 2003).

Complementary strategies involving better inactivation techniques and detection methods in the human food supply would therefore be an attractive way of BSE surveillance. In order to achieve this objective, it is imperative to understand not only the biochemistry of abnormal prion protein but also the normal isoform of prions (PrP^C). A key obstacle to developing these strategies is the availability of PrP^C in sufficient quantities in order to conduct studies to enhance our understanding its properties. Even though the availability of PrP^C in the brain is quiet high when compared to other tissues, its representation is less than 0.1% of the total proteins in the central nervous system (Riesner, 2003). Purification of PrP^C from a tissue source has often led only to

disappointment, as it is extremely difficult to obtain large amounts of the protein (Mehlhorn et al. 1996). For example, from 100 hamster brains only a few micrograms of PrP^C could be extracted using the optimal procedure (Riesner, 2003). The availability of large quantities of purified recombinant PrP^C should therefore facilitate the study of the structural properties of PrP^C. In the past, scientists have produced recombinant PrP^C of various species in a variety of expression system including mammalian, baculovirus, vaccine virus, bacteria, and yeast. However, considerable amount of problems were faced during the expression and purification of recombinant PrP because of low expression, insolubility and protein instability during the process (Baron et al., 1999). Syrian Hamster (SHa) PrP full-length mature protein 23-231 were highly expressed using a bacterial vector and the maximum expression was achieved for the region 90-231 which corresponds to the protease resistant core PrP 27-30 (Mehlhorn et al. 1996). Baron et al (1999) overexpressed PrP^C from sheep in large amounts in *E. coli* using amino terminal fusion with glutathione-S-transferase (GST) and carboxy-terminal fusion with hexahistidine sequence with varying affinity of each of the expressed protein on Western blots (Baron et al., 1999).

Recombinant sheep prion protein (RecShPrP^C) is also produced commercially by firms like AJ Roboscreen GmbH, Leipzig, Germany who has produced histidine-tagged full-length mature part of sheep PrP (25-234) expressed in *E. coli* BL21, solubilized from inclusion bodies in 6 M guanidine-HCl, and purified by Ni(II)-nitriloacetate agarose chromatography. However commercially available products like this costs more than $3,000.00 per milligram which would be very expensive for research needing large quantities of PrP.

The objectives of this study was to overexpress the sheep prion protein as a fusion using the carboxy-terminal hexahistidine tag, solubilize it in 8M guanidinium chloride and purify it using a Ni-column affinity chromatography. Also the expressed protein was detected using a Western blot assay. Scrapie is the spongiform encephalopathy found in sheep and goats. We decided to use normal sheep prion protein for our expression studies for two reasons: (1) Sheep PrP (ShPrP^C) is one of the most studied and well understood prions proteins, and (2) the ShPrP^C sequence significantly differs from human PrP, implying that considerable barrier for the transmission of scrapie or ShPrP^C to humans. It is highly unlikely for the scrapie to be transmitted to human beings because scrapie is common to sheep and the CJD occurs in one in millions.

CHAPTER II

REVIEW OF LITERATURE

2.1. Prions – An Overview

The early history of prion molecule goes back to the study of the physical and bio-chemical structure of the scrapie infectious agent. Unlike a virus, prions demonstrate highly enigmatic properties like resistance to high temperatures and other physical and chemical treatments, absence of particles when seen through an electron microscope and no immune response during infection (Alper et al., 1966). Alper et al (1966) concluded from their studies that scrapie agents have characteristics more similar to proteins than a virus as they were too small (50-150 kDa) to be a virus. The mystery of scrapie agent was later resolved by Prusiner who not only did biochemical and physical studies but also performed a lot of enzymatic studies on the scrapie agent and also coined the term "prion" (pronounced *pree-on*) derived from **pro**teinaceous and **in**fectious, as they were different from viruses, plasmids and viroids (Prusiner, 1982). He noted two key results from his experiments: (i) the scrapie agent has no effect on procedures that modify or destroy nucleic acids, and (ii) the procedures that modify or destroy proteins inactivate scrapie agent (Prusiner, 1982). Prions are mysterious infectious particles which cause a set of neurodegenerative diseases caused by a completely novel mechanism (Prusiner, 1998). Prion diseases in humans may occur as genetic, acquired or sporadic forms and in all situations, the cellular prion protein (PrP^C) which is constituent of all mammalian cells is converted into an abnormal form designated PrP^{Sc} followed by accumulation of PrP^{Sc} in neuronal and/or lymphoid tissues (Riesner et al., 1983).

Creutzfeldt-Jakob disease (CJD) is an example of prion disease in humans with rapidly progressing dementia eventually leading to the death of the victim in 3-4 months whereas BSE in cattle and scrapie in sheep and goats are ataxic illnesses (Collinge and Rossor, 1996; Prusiner, 1998).

2.2. Terminology

Amyloid

The term Amyloid is used for deposition of pathologic protein filaments. These filaments exhibit green-gold birefringence when stained with Congo red and seen under polarized light. Amyloids formed in a prion disease are called cerebral amyloids (Prusiner et al., 1983).

Amyloid Plaques

Amyloid plaques are formed when protein filaments aggregate in the extra cellular spaces of brain (Prusiner et al., 1987).

Chronic Wasting Disease

The transmissible neuro-degenerative disease found among mule deer and elk presumably caused by prions. The hallmark of this disease is also the formation of amyloid plaque in the brains of the diseased animal (Williams et al., 1982).

Cosmid

A hybrid plasmid that contains cos sites at each end. Cos sites are recognized during head filling of lambda phages. Cosmids are useful for cloning large segments of foreign DNA (up to 50 kb).

Creutzfeldt-Jacob Disease

Creutzfeldt-Jacob Disease (CJD) is a transmissible neuro-degenerative disease in humans caused by prions. Patients with CJD exhibit a progressing dementia with myoclonus and a characteristic electroencecephalgram (ECG) showing pseudo periodic sharp complexes eventually leading to death of the patient in 3-4 months (Collinge and Rossor, 1996). CJD occurs at the rate of one per million population annually across the world and the onset of disease occurs at an average age of 65 years and is rarely seen below the age of 35 years (Collinge and Rossor, 1996).

Gene

Structurally, a basic unit of hereditary material; an ordered sequence of nucleotide bases that encodes a product (this product could be just RNA like rRNA or finally coding for a protein). The gene includes, however, regions preceding and following the coding region (5' UTR and 3' UTR) as well as (in eukaryotes) intervening sequences (introns) between individual coding segments (exons) (Biochemistry, 2002).

Leader Sequence

It is the sequence at the 5' end of an mRNA that is not translated into protein. It is also the length of untranslated mRNA from the 5' end to the initiation codon AUG. It is the polynucleotide sequence between promoter and structural gene, necessary for correct transcription of DNA into mRNA (Collinge and Rossor, 1996).

Meat and bone meal (MBM)

Meat and bone meal (MBM) is the dried and rendered product from mammal tissues which is considered a high source of protein. It does not contain horn, hair, hide trimmings, manure, stomach contents, added blood meal or poultry by-product. In the

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year 1988 United Kingdom imposed a ban on the use of meat and bone meal in the animal feed (Taylor et al., 1994).

Open reading frame (ORF)

A section of a sequenced piece of DNA that begins with an initiation (methionine ATG) codon and ends with a nonsense codon. ORFs all have the potential to encode a protein or polypeptide, however many may not actually do so (Collinge and Rossor, 1996).

Plasmid

Plasmid is a small infectious particle which is composed of circular double-stranded DNA and without any protein in it. They are usually found in bacteria and sometimes in eukaryotic organisms with an average size anywhere between 1 – over 400 kilo base pairs (kbp) (Holmgren and Lefers, 2002).

Prion protein

Prion proteins are the protease resistant glycoprotein core obtained while enriching fractions from Syrian hamster brain for scrapie infectivity and is designated as PrP 27-30 which has an apparent molecular weight of 27,000 to 30,000. PrP^C is derived from prion protein. PrP 27-30 is obtained from digesting the PrP $33-35^{Sc}$ with proteases which has an apparent molecular weight of 33,000 to 35,000 on an SDS-PAGE gel. The superscript Sc is used to differentiate it from its cellular (C) isoform PrP $30-35^C$ (Prusiner, et al., 1987).

Prion protein Gene

The prion protein gene has been identified in many organisms including humans (Chesebro et al., 1985). The human prion gene is designated PRNP and is located on the short arm of chromosome 20 (Sparkes et al., 1986).

Scrapie

Scrapie is the prototype for prion diseases. It is a transmissible neuro-degenerative disease in sheep and goats caused by prions characterized by prolonged incubation periods preceding the onset of critical illness. Other neurological symptoms include ataxia, pruritis and incoordination (Prusiner, 1994). Like other prion diseases, it is progressive and fatal in nature.

Specified Risk Materials

Specified Risk Materials (SRMs) are tissues in BSE-infected cattle, which have been shown to contain the infective agent and transmit the disease. SRMs in all cattle are tonsils and distal ileum of the small intestine. Brain, skull, eyes, trigeminal ganglia, spinal cord, dorsal root ganglia (DRG), vertebral column, excluding Vertebrae of the tail Transverse process of the thoracic and lumbar vertebrae are SRMs in cattle that are 30 months and older (Turk et al., 1988).

Tranformation

It is the process of introduction of an exogenous DNA into a cell, causing it to acquire a new phenotype.

Variant Creutzfeldt-Jacob Disease (vCJD)

The year 1994 saw the emergence of a new form of CJD in United Kingdom which was distinctive from classical CJD; young age at onset, psychiatric and sensory symptoms and long disease course. All the cases of this variant CJD (vCJD) is homologous at codon 129 for methionine (Will et al., 1996). The prion found in BSE and in vCJD has the same physical and chemical characteristics indicating the fact that they have common sources. Hence, it is believed that vCJD is caused by the consumption of infected beef products (Hill et al., 1997).

Vector

A vector is a DNA molecule known to replicate autonomously in a host cell, to which a segment of DNA may be spiced in order to bring about it replication; i.e., a plasmid or a temperate-phage DNA (temperate phage DNA is incorporated into host genome as prophage which replicates with the host bacterium, whereas virulent phage lyses the host cell).

Viroid

Viroids are small infectious pathogens which are entirely composed of low-molecular weight RNA. Viroids use the enzymatic machinery of the host for reproduction. Viroids have been found only in plants (Prusiner, 1994).

Virus

Viruses are a small infectious pathogen composed of one or more than one nucleic acids and covered with coat of protein. Some viruses are also coated with lipid and carbohydrates. Viruses direct the synthesis of their progeny by using its nucleic

acids and making use of the enzymatic machinery of the host (Prusiner and McKinley, 1987).

2.3. The Prion Hypothesis

A large amount of research data supports the 'Protein only' hypothesis, even though the exact nature of the physical characteristics of prion is not known as first described by Griffith (1967) and later in a detailed manner by Prusiner (Prusiner, 1982). According to Prusiner, the transmissible agent does not have a nucleic acid and converts the rest of the normal protein into the abnormal protein PrP^{Sc} (Prusiner, 1982). This proposal is supported by recent findings showing that mouse PrP^{c} expressed in yeast and neuronal tumor cells can be changed into a form resembling very closely to the naturally occurring mouse prions (Sims, 2000). It is not beyond doubt that these transgenetically produced prions would infect mice in the absence of a virus or virino. If proved so, it would remove the doubt among the skeptics who still believe the virus or virino concept behind the TSEs (Riesner et al., 1983). Now there are other reasons why scientists have abandoned the virus or virino hypothesis. Firstly, the size of the prion protein is much smaller than any virus (Riesner et al., 1983). Secondly; TSE scientists could not yet identify a scrapie specific nucleic acid (Riesner et al., 1983). The presence of different strains of prions within the same species have raised questions about the protein-only hypothesis. It is assumed that nucleic acids would explain the occurrence of different strains as it provides the probability of mutations. However the different physiochemical properties of infectious protein (PrP^{Sc}) like glycosylation and proteolysis both endogenous and exogenous, explains the strain differences in scrapie (Collinge et al., 1996).

2.4. Prion Protein (PrP) genetics and expression

2.4.1. Prion protein gene structure

The *prnp* gene, which codes for the prion protein is present in most mammals and is highly conserved across species (Van et al., 2003). The entire open reading frame (ORF) of the PrP gene of all the mammals and bird species tested so far lies on a single exon (Prusiner, 1989). In sheep, rat and cattle, there are three exons for the PrP^C gene and the exon 3 has the ORF, which is analogous to exon 2 of the SHaPrP (Syrian Hamster) gene. The exon 1 and exon 2 of the SHaPrP gene is separated by a 10-kb intron where exon 1 has the 5' untranslated leader sequence and exon 2 has both 3' untranslated leader sequence and the SHaPrP gene (Prusiner, 1989).

The sequence comparison study of SHa- and MousePrP (MoPrP) genes reveal that the promoter regions of both species have multiple copies of G + C rich repeats and they also lack the TATA box (in eukaryotes TATA box helps the RNA polymerase to bind to the promoter sites and begins transcription). These G + C repeats in these species may function as a binding site for the transcription factor Sp1 (Basler et al., 1986). The mapping of HuPrP to the short arm of chromosome 20 is homologous to MoPrP mapped to chromosome 2 which supports the existence of PrP gene before speciation of mammals (Stahl et al., 1993). The ORFs of PrP genes of many mammals and other class of organisms including humans have been sequenced and they all encode prion proteins of approximately 250 amino acids with N-terminal signal peptides (Kretzschmar et al., 1986). The N-terminal portion of all the PrP ORFs also have a series of glycine rich regions. The C-terminal region of all the PrP ORFs encode for a hydrophobic peptides

which later is presumable replaced with a GPI anchor and it also carries two consensus sites for glycosylation of asparagine and cysteines (Prusiner, 1989).

2.4.2. Prion protein expression

The mRNA of PrP^C is expressed constitutively in the brain and many other cell types of all the adult mammals however, it is highly regulated during development (Basler et al., 1986). Levels of PrP mRNA and choline acetyltransferase in septum increased during the development stage. In situ hybridization studies shows that PrP is expressed in the highest levels in neuronal cells. In some parts of the brain, PrP is expressed at an early stage (Basler et al., 1986).

Expression of PrP^C was determined in the brain by immuno-histochemistry and by histo-blotting of both infected and uninfected brains. Immunostaining of PrP^C in SHa brain was very prominent in amygdala region of brain whereas immunostaining of PrP^{Sc} was less intense in the same region. Similar relationships between PrP^C and PrP^{Sc} were observed in other parts of the brain like stratum radiatum and stratum oriens of CA1 region of hippocampus. In contrast, PrP^{Sc} accumulated in the medial habenular nucleus, the medial septal nuclei, and the diagonal band of Broca; in contrast, these areas were virtually devoid of PrP^C.

Expression studies in transgenic (Tg) mice indicated that the normal PrP play a crucial role in the transmission and pathogenesis of prion diseases, however they also noted that PrP^{Sc} is an equally important part of the prion particle (Prusiner, 1991). In other knock out (KO) studies of mice conducted by Prusiner et al, they observed that *prnp* gene deficient (*prnp*$^{0/0}$) mice were resistant to prions. Brain extracts from these

mice were extracted at different time points and were bio-assayed and found out that there is no infectivity in the brains of the *prnp*[0/0] mice.

2.5.   Chemical properties of PrP and recombinant PrP

The single exon on the gene *prnp* encodes for a 256 – 264 amino acids (AA) precursor of an approximately 28 kDa product which is further processed when a signal peptide of 22- to 24-AA is cleaved from both C- and N- terminals leading to mature protein of 231 to 253 AA consisting of approximately 209-210 residues(Riesner, 2003).

Prusiner et al. (1983) showed that after limited proteolysis of the abnormal isoform of PrP, the amino terminus is truncated to form PrP 27-30, which is composed of ~142 amino acid residues.

During trafficking of the peptide out of the cell, the signal peptide 1-22 AA is cleaved and a glycosyl-phosphatidylinositol (GPI) is anchored at the site of the peptide 232-253. Prion protein is anchored by GPI on the cell surface (Turk et al., 1988). Asparagine residues at 181 and 197 carry highly branched glycosylation with sialic acid substitution. On a western blotting assay followed by SDS-PAGE separation of the PrP, three forms of glycosylation can be seen based on glycolsylation-unglycosylated, with one glycosyl-, and with two glycosyl-groups. A disulphide bridge is formed between Cys179 and Cys214

The complete coding sequence of a sheep (*Ovies aries*) *prnp* gene is shown below in Fig A (Heaton,M.P., Leymaster,K.A., Clawson,M.L. and Laegreid,W.W., Animal Health Research Unit, U.S. Meat Animal Research Center, Box 166, State Spur 18D, Clay Center, NE, personal communication). The original translation product is a

256 amino acid peptide and the N-terminal signal peptide is 24 amino acids long. The C-terminal peptide 235-256 is replaced by a GPI anchor. Therefore, PrP 25-234 is the major prion protein in sheep.

Figure A

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MVKSHIGSWILVLFVAMWSDVGLC  KKRPKPGGGWNTGGSRYPGQGSPGGNRYPPQGGGGWGQPHGGGWGQP

HGGGWGQPHGGGWGQPHGGGGWGQGGSHSQWNKPSKPKTNMKHVAGAAAAGAVVGGLGGYMLGSAMSRPLI

HFGNDYEDRYYRENMYRYPNQVYYRPVDXYSNQNNFVHDCVNITVKQHTVTTTTKGENFTETDIKIMERVV

EQMCITQYQRESQAYYQRGAS  VILFSSPPVILLISFLIFLIVG
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(NCBI ACCESSION DQ345757) (Heaton,M.P., Leymaster,K.A., Clawson,M.L. and Laegreid,W.W., Animal Health Research Unit, U.S. Meat Animal Research Center, Box 166, State Spur 18D, Clay Center, NE, personal communication).

2.6. Prion Detection

2.6.1. Introduction

Prions are infectious proteins, which act by changing the structure of the normal prion protein (PrP^C) into an abnormal protein (PrP^{Sc}) in the host (Sailer et al., 1994). Even though this hypothesis is widely received among the scientific community, no direct and unquestionable proof is available (Cheesbro, 2002). Prusiner separated a protease and a heat resistant protein from the brain of a hamster infected by scrapie in the year 1982, and he termed it as PrP^{Sc}, which closely resembled the infectious agent (Cheesbro, 2002). Charles Weissmann at the Institute of Molecular Biology in Zurich (Basler et al., 1986) and Bruce Chesebro et al (1985) independently identified the gene *PmP* that encodes PrP^C. The activity of the gene *PmP* is seen not only in brain but also in other parts of the body in both scrapie infected and non-infected sheep. The *PmP* gene

was removed from the mouse genome to examine the effect on the development of the mouse but to the surprise of the scientists, it did not prevent the normal development of the mouse, but it still exhibited complete immunity against scrapie (BuÈeler et al., 1992; BuÈeler et al., 1993; Sailer et al., 1994).

The nervous system is the only organ system, which can be attacked very severely by the TSE infection. This also applies to other prion diseases such as Creutzfeldt-Jakob disease, Gerstmann-Sträussler-Scheinker Syndrome, Kuru and fatal familial Insomnia and to all known prion encephalopathies of animals (Aguzi and Heppner, 2000). However, it is evident that prions can attack organs other than the nervous system as seen in the spleen of scrapie-infected mice (Aguzi and Heppner, 2000).

2.6.2. The Prion Conversion Reaction:

Molecular studies reveal that both PrP^C and PrP^{Sc} are glyco-proteins and they weigh approximately 35KDa. PrP^{Sc} is an abnormal isoform of the normal protein, which is anchored to the cell membrane by a glycosylphosphatidylinositol (Stahl et al., 1993). Both the proteins are chemically same, but differ in the conformation. Though the same gene codes both isoforms, they exhibit different physiochemical properties because of the different post-translational processes, which happens once PrP^C reaches the extra cellular location (Baslet et al., 1986). The accumulation of PrP^{Sc} in the brain is a typical characteristic of all the prion diseases. The study of the 3D structure of PrP^C has given several insights and speculations on the mechanism of the conversion of PrP^C into PrP^{Sc} in familial spongiform encephalopathies (Pan et al., 1993). However, it has failed to give proper insight into the structure of the high-resolution PrP^{Sc} isoform. This study of 3D structure as seen in the following figure revealed that PrP^C has a high α-helix content of

42% and a very low β-sheet content of 3%, whereas PrP^{Sc} has 21% α- helix and 54% β-

PrP^C   **PrP^{Sc}**

Fig B

sheet content (Pan et al.,1993). In order to explain the conversion of a normal PrP^c

protein to an abnormal PrP^{Sc} from a misfolded PrP^{c,} two models have been hypothesized:

(i) the template assistance model which is also known as the 'refolding' model and (ii)

the nucleation-polymerization of 'seeding' model (Aguzi and Heppner, 2000). The first

model talks about the high activation energy barrier, which prevents the conversion of

PrP^C to PrP^{Sc} spontaneously at detectable rates. When PrP^{Sc} is introduced exogenously, it

converts the PrP^C into PrP^{Sc} (Aguzi and Heppner, 2000). This might involve a lot of

unfolding and refolding which explains the high energy and might involve an enzyme or

a chaperone.

        In the second model, the two isoforms are in equilibrium strongly favoring the

PrP^C (Telling et al., 1995). When the PrP^{Sc} combines with a crystal like PrP^{Sc} aggregate,

the complex is stabilized and hence more of this complex is formed. The PrP^{Sc} aggregate

here acts as a seed for the nuclear-polymerization reaction (Jarrett and Lansbury, 1993).

2.6.3. Immunohistochemical Detection of Prions

Researchers in the University of California - San Francisco have developed a highly sensitive test to detect the presence of infectious prion proteins (Safar et al., 2002). This test makes use of the high affinity antibody, which precisely detects even the smallest amount of infectious protease resistant prion proteins present in the meat. This method is known as Conformation-dependent Immunoassay (CDI). For the production of this bioassay, a genetically engineered mouse for the over expression of bovine prion protein is injected with prions from an infected BSE (Bovine Spongiform Encephalopathy) cow. This transgenic mouse with the capability to express the bovine prion protein is more sensitive to a prion attack from the infected cattle than a normal mouse. Scientists claim that the assay produced in this kind of a genetically engineered mouse can detect 10,000 times more infectious proteins than a normal mouse in a standard assay (Safar et al., 2002). The older immunological techniques detect only a portion of the PrP^{Sc} in cattle.

Immunohistochemistry has become a standard tool for researchers in diagnostic-pathology. The objective with regard to the prion detection is to prove the presence of infectious protein in the body of an animal because most of the time, it is very difficult to differentiate the infected part of the body from the healthy parts. Researchers at Prusiner's Lab at UCSA are very confident about the performance of the new assay they have developed (Safar et al., 2002). According to Safar (2002), they can use the CDI technique in cattle, to reduce the incidence of exposure of humans to prions.

2.6.4. Conformation-Dependent Immunoassay

About 20 years ago, researchers in Prusiner Lab developed the standard technique, which involved the enzyme protease being used to inactivate the normal prions, found in brain. Study has revealed that this protein is used for the signal transduction inside the cell (Chesebro, 2002). Scientists highlight the remaining protease resistant PrP^{Sc} with fluorescently lit antibodies that reacts with these resistant proteins (Farquhar et al., 1989; Safar et al., 2002). However, the problem with this technique is that, a large amount of the abnormal protein is protease sensitive and is undetected by the antibody. Therefore, scientists are not able to get a good idea of the actual amount or exact number of infectious particles present (Safar et al., 2002).

The new technique involves the use of a high-affinity antibody, which identifies the PrP^{Sc} by its structure thus helping the scientists to get the actual amount of these proteins. This does not make use of the protease enzyme. At the onset, an infectious tissue extract in its natural form is treated with the high-affinity antibodies and the reactivity is measured. Then the infectious protein is treated with certain chemical so that they are unfolded and the region hidden earlier is exposed. It is then treated with the antibodies and the reactivity is measured. The degree of reactivity of antibody is much higher in denatured protein than the native one. The ratio of native diseased prion protein to the denatured one gives the actual amount of PrP^{Sc} in the tissue (Farquhar et al., 1989).

2.6.5. Immuno-double staining

Scientists have identified two techniques to study and identify the nature of PrP^{Sc} molecule. In the first technique, two different antibodies are used to immunostain two serial tissue segments one on each side; one antibody identifying the cellular component

and other binding the PrP^{Sc} (Noorden, 1986). Secondly, a similar immuno-double staining technique using two different antibodies on a single tissue can be used on a single tissue segment (Hancock, 1984). A color-coding is required in this kind of a labeling technique to identify and differentiate the antigen of interest. The shortcoming with the first technique is the risk of a similar kind of tissue structure or cell is not being present in the adjacent segments. Another disadvantage of the immuno-double staining technique is the incidence of cross-reactions between two individual methods of detection (Noorden, 1986).

2.6.6. Western Blotting

Western Blotting is one of the most often used methods for an immunoblotting technique. With this technique scientists can detect the protein of interests using specific antibodies with very high accuracy. Proteins, which are separated based on the molecular weight, are transferred from a Sodium Dodecyl Suphate (SDS) polyacrylamide gel to a nitrocellulose membrane. The protein of interest is thereafter detected using specific antibodies (Towbin et al., 1979). The detection method is similar to the one used in immunohistochemistry: the protein is first bound to an antibody and after that a secondary antibody is incubated with the primary antibody-antigen complex (Thuring et al., 2002). Conventional staining, chemiluminescence, autoradiography or flurography are used for observing the antigen-antibody complex depending on the method of labeling the secondary antibody (Towbin et al., 1979). In prion related diseases, western blotting has been in use for detecting the protease resistant protein in brain tissues of cattle, sheep (Katz et al., 1992), mice , humans and hamsters. This technique is also used to test the presence of prions (PrP^{Sc}) in Lymphoid tissues. Scientists have identified

the use of Western Blotting in detecting BSE and scrapie in sheep on a large scale. Upon treatment with Western Blotting technique, the PrP^{Sc} separates into 3 layers which is manifested as three bands on the blot: nonglycosylated PrP^{Sc}, monoglycosylated PrP^{Sc} and diglycosylated PrP^{Sc} (Collinge et al., 1996; Thuring et al., 2002). Each of these fractions has different molecular weights. Based on the ratio of their molecular mass or the Glycotype pattern, scientists are able to identify the different strains of scrapie (Thuring et al., 2002).

2.6.7. Immuno-PCR

Sano et al (1992) developed a highly sensitive technique for antigen detection called Immuno-PCR which makes use of the molecular recognition property of antibody with the DNA amplification capacity of PCR (polymerase chain reaction). This technique could detect nucleic acids at femto grams levels of molecules using PCR (Niemeyer et al., 1997). Immuno-PCR is similar to an enzyme-linked immunosorbent assay (ELISA) and western blotting assay however it is much more sensitive in detection than both. Unlike ELISA, immuno-PCR makes use of an antibody instead of an enzyme. A primary antibody binds to the specific antigen and a biotinylated secondary antibody made against the primary is incubated together. The whole complex is then reacted with streptavidin or neutravidin that has a natural affinity towards biotin, which is further incubated with biotinylated DNA that undergoes amplification in a PCR reaction. This method was improved by the use of real-time PCR that would eliminate all the post-PCR analysis. Gofflot et al (2005) used this technique to detect PrP^{Sc} with 100% specificity with a detection level of at least 10-fold lower than the conventional techniques. This method could possibly be used to detect the very small amount of PrP^{Sc} deposits in

tissues and body fluids and hence be used for monitoring effective decontamination and sterilization methods.

2.6.8. Conclusion

Ever since Prusiner identified prions as the cause of many neurodegenerative human diseases, scientists around the world have been working on a technique, which would be highly sensitive towards these infectious proteins. So far there are no efficient tests available to detect TSEs completely. CDI technique discovered at Prusiner Lab would be a great tool towards the detection of all prion related and many other neurodegenarative diseases, which can be understood better.

2.7.   Degradation of Prions in Meat

Several incidents of prion infectivity among humans (Creutzfeldt–Jakob disease or CJD) were reported in Britain and France in late 80s soon following an epizootic outbreak of bovine spongiform encephalopathy among cattle, which suggested scientists a connection between the two diseases (Will et al., 1996; Bruce et al. 1997). As mentioned earlier, these set of diseases are extremely fatal in both animals and humans and hence require very effective therapeutics (Stahl et al., 1993). Scientists have discovered a number of chemicals, which have shown a minimal effect in the degradation of these infectious protein and especially in the propagation inside the tissue of an infected animal. Some of them are anthracycline, sulfated polyanions, amphotericins, Congo red dye and antibiotics which exhibit a modest curative effect on TSEs but are unable to completely degrade the preexisting prions (Supattapone et al., 1999).

2.7.1. Destruction of Prions

The wide array of degradation and inactivation techniques that have proven effective for conventional microorganisms have failed to inactivate the agents that cause prion related diseases in animals and humans (Kimberlin et al., 1983; Brown et al., 1986; Taylor et al., 1997; Taylor, 1999b; Taylor, 2002). Some inactivation techniques, which were thought to be very effective for the degradation of prions, are now found to be only partially effective. Such methods involve the exposure of the infected tissues to 1M Sodium Hydroxide for one hour at 37°C and autoclaving at 134-138°C (Porous-load technique) or autoclaving at 132°C (gravity displacement method) (Taylor et al., 1999a). The other recommendation of the use of chlorine (20,000 ppm) in combination with sodium hypochlorite proved to be effective in decimating the infectious agent, however it renders the meat product inedible (Kimberlin et al., 1983; Taylor et al., 1997). A number of research data indicates that, the prions can be completely destroyed by using the above mentioned techniques simultaneously or in a series at an autoclaving temperature of 121°C (Taylor, 1999b). However, it has been found that the presence of high amount of lipid concentration in the infectious tissues may lower the efficacy of autoclaving of these agents due to the hydrophobic characteristic of PrP^{Sc} protein (Appel et al., 2001).

2.7.2. Effect of Temperature and Pressure

Dr. Paul Brown and his co-researchers at the National Institute of Health, Bethesda, MD, (Brown et al., 2003) have succeeded in significant reduction of the infectivity of prions in processed meats using a combination of high pressure and temperature without affecting the texture and taste of the food. They used the scrapie infected brain paste of 263K strain of hamster-adapted scrapie mixed with hotdogs,

which was subjected to high pressure and temperature. Several short pulses of pressure ranging from 690 to 1200 MPa (up to approximately 100,000 psi) were applied to the hot-dog mix at a temperature of 121 to 137 ^{0}C. Western Blot analysis of PrP^{Sc} showed that the high-pressure treatment achieved a reduction in the infectivity levels by 10^3 to 10^6 mean lethal doses (LD_{50}) per gram of the homogenate mix. Maximum inactivation of 4-6 log LD_{50} per gram was achieved at pressures of 1,000 MPa and 1,200 MPa. High pressure had been in used for more than 100 years for decimating the microbial population in food products, however this technique got more popularity with the advent of more sophisticated high pressure equipments (Brown et al., 2003). Inactivation of the bacterial, viral, and fungal pathogens in the present day would have better results under hyperbaric and high temperature conditions with the exception of few like *Listeria* and λ phage, which has inactivation temperatures near 0^0C. Dr. Brown and his team are continuing their work on the degradation of prion using high pressure technology especially using different combinations of temperature, pressure and pulse rates and also in other TSE strains like natural and experimentally adapted BSE, nvCJD (Brown et al., 2003).

2.7.3. Elimination of prions by branched polyamines and implications for therapeutics

Supattapone et al (1999) reported that the infectious prions (PrP^{Sc}) inside a scrapie-infected neuroblastoma (ScN2a) cell culture can be eliminated by the non-cytotoxic concentrations of branched polyamines like polyamidoamide dendimers, polypropyleneimine, and polyethyleneimine (Supattapone et al., 1999). The degradation of PrP^{Sc} in these cells depended on the concentration and time the cells were exposed to

the branched polyamines (Supattapone et al., 1999). The exposure of ScN2a cells to these branched polymers for a period of one week reduced the PrP^{Sc} in these cells into undectable amounts. This condition was maintained for a period of three weeks even after the withdrawal of the branched polymers from the culture (Supattapone et al., 1999).

The structure-activity relationship (SAR) study indicates that the presence of the regularly placed primary amino structures on the polyamines is the key for the elimination of the infectious protein PrP^{Sc} from the cells. A meticulous study of these structure-activity analyses suggests two to three mechanisms explaining the elimination process of PrP^{Sc}. One of them suggests the binding of ligands present on PrP^{Sc} with the branched polyamines causing the opening up of negative moieties, which is followed by a conformational change (Supattapone et al., 1999). This change is favored by the presence of an acidic environment. The use of the heat resistant protease enzyme had a considerable effect on the scrapie-infected cells when the culture was first treated with branched polyamines at low pH (Supattapone et al., 1999). Experiments suggest that acidic environment causes the PrP^{Sc} to unfold into monomers and as a result increases the concentration of α-helices. Another explanation is that the polyamines chelate the negatively charged moieties that are bound to PrP^{Sc} needed for protease resistance; however, they are released once the conformational change is complete under the acidic environment. Clearly it indicates that more work is needed to understand the mechanism behind the action of polyamines on the PrP^{Sc}.

2.7.4. Enzymatic Degradation of Prion Protein

Researchers at the North Carolina State University in association with the scientists from the Netherlands used the enzyme Keratinase derived from *Bacillus liceniformis* to successfully degrade the infectious isoform PrP^{Sc} of the normal cellular prion protein (Langeveld et al., 2003). As mentioned earlier, this infectious protein is highly resistant to common treatments like boiling and activities of enzymes like proteinase K under normal conditions. This method of degradation was aimed at decontamination of laboratory and medical equipment, which come in contact with the infectious materials. The normal way of decontaminating the infected equipment in the lab is by boiling with 1% sodium dodecyl sulfate (SDS) with a reducing agent or treating it with chlorine, sodium hydroxide or formic acid (Taylor et al., 2000). Since these methods have failed to yield the right results and also because of the harsh nature of these techniques towards the food product, scientists are looking for better alternatives. As mentioned before, researchers at The National Institute of Health, MD, have used a combination of various treatments such as heat, temperature and pressure to mitigate the infectivity of prions significantly in processed foods (Brown et al., 2003). Study on the use of enzymatic degradation of these infectious agents in conjunction with some of the conventional techniques like temperature and pressure has been shown to work better in the degradation process of prions, as it disrupts some of the hydrogen bonds, facilitating the solubilization and the digestion process by the enzymes (Laemmli, 1970). This group of researchers also tested the effect of heating the infected homogenate in the presence of a detergent (N-Laurosylsacosine) on the prion degradation (Langeveld et al., 2003). The test results and the western blot analysis indicated the reduction of the rogue PrP to immunochemically undetectable levels (Langeveld et al., 2003). So these tests convinced

the scientists that it is possible to destroy completely PrPSc in BSE infected tissues and such techniques has the potential to bring about complete inactivation.

2.7.5. Conclusion

No effective treatments or techniques are available for inactivating the prions in the foodstuffs especially in processed meats. However, the researchers at the North Carolina University have succeeded in inactivating prions in vitro or in test tubes to undetectable levels, which would be helpful in decontaminating medical and laboratory equipments (Langeveld et al., 2003). Use of high pressure-temperature combination also has shown a reduction in prion in infected tissues. Prusiner and his co-workers explained the action of branched polyamines on prions, which could be used in finding a therapeutic cure for prion related diseases. The major concern from a food-processing standpoint would be to find a practical and commercial solution to eliminate prions from the food sources without effecting the texture and taste of the food. The objectives of this study were to develop a protocol for the over-expression of sheep prion protein in a bacterial cell line and its large-scale purification using a Ni-NTA column. It would thus overcome the difficulties in extracting and purifying normal prion protein from tissue sources for the structural and biochemical studies.

CHAPTER 3

PRODUCTION OF RECOMBINANT SHEEP PRION PROTEIN (RecShPrP^C) AND ITS DETECTION USING WESTERN BLOTTING AND IMMUNO-PCR

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ABSTRACT

The objectives of this study were to over-express the sheep prion protein (RecShPrP^C) using a bacterial vector fused with a C-terminal *6XHis*-tagged fusion protein for large-scale purification using a Nickel-Nitrilotriacetate column chromatography. The prion protein gene was PCR amplified using sheep genomic DNA and only the functional prion polypeptide (PrP 25-234) was sub-cloned into pET102/D-TOPO plasmid after removing the sequences corresponding to the signal peptides from both N-terminal and C-terminal ends. The integrity of the cloned PCR product as well as the *His*-tagged fusion protein and its orientation within the vector was determined by DNA sequencing and upon confirmation was induced with isopropyl-beta-D-thiogalactopyranoside for protein expression. The expressed proteins were solubilized by addition of 8M guanidinium chloride in lysis buffer and purified using adsorption to a Nickel-Nitriloacetic^{++} metal affinity resin. Purified proteins were detected at levels as low as 10μg using Western Blotting. Immuno – PCR is a recently described method for ultra-sensitive antigen assay, which was able to detect the RecShPrP^C as low as 200 femto-grams. Production of RecShPrP^C protein by this method could be used to study solubilization processes that fractionate muscle proteins. Successful separation of myofibrillar and sarcoplasmic proteins from abnormal prion proteins may enhance the

safety of meat products that would eventually be rendered and used for non-mammalian animal feed or fertilizer.

Key Words: Expression, Fusion Protein, His tag

INTRODUCTION

Scientists around the globe have shown intense interest in a group of transmissible neuro-degenerative diseases among which are spongiform diseases of cattle, scrapie of sheep, and Creutzfeld-Jacob disease of humans. The agents responsible for transmitting these diseases are called prions, which are infectious proteins (Prusiner, 1994). According to Prusiner's 'protein-only' hypothesis (Prusiner, 1982) the normal prion protein (PrP^C) is converted to the abnormal isoform (PrP^{Sc}) by post-translational alterations to its secondary structure (Pan et al., 1993; Weissman, 1995). Although chemically identical to PrP^C, sensitivity to proteases, different cellular distribution and altered solubilities all differentiate PrP^{Sc} from its parent isoform (Prusiner et al, 1983; Stahl et al 1993). In order to understand the biochemistry and molecular mechanism of prions, it is key to study the structural properties of PrP^C and PrP^{Sc}. Attempts have been made in the past to extract and purify PrP^C from tissues and bacterial expression systems, however very low expression and yield has hindered the process (Caughey et al, 1988; Scott et al, 1988). Spectroscopic and NMR studies have been carried out successfully on hamster and mouse PrP fragments that were a part of the infective core of PrP^{Sc} (Mehlhorn et al, 1996).

To overcome these issues, we expressed the full-length mature sheep PrP (25-234) in a bacterial system fused with a polyhistidine extension (His-PrP) at the C-terminal end. Sheep prion protein was selected because it was safer to deal with and is a much studied protein. The His-PrP was recovered from inclusion bodies with guanidinium chloride, purified by Nickel-Nitriloacetic^{++} affinity chromatography and confirmed using Western Blotting and Immuno-PCR. Our future work includes the tranfection of a mammalian cell with the prion gene ORF and expression of the protein for structural studies.

MATERIALS AND METHODS

Materials

Polyvinyl-diflouride (PVDF) membrane, medium thick blotting paper, 2X Laemmli Sample buffer, and Proteinase K (200 µg/ml), was purchased from Sigma (St. Louis, MO). Sucrose was obtained from United States Biomedical Corporation (Cleveland, OH) and agarose gel was obtained from MP Biomedical (Irvine, CA). Polypropylene plate were obtained from BD Biosciences (Franklin Lakes, NJ). Plasmid extraction material was obtained from Qiagen (Valencia, CA). The protein expression and purification material was bought from Invitrogen (Carlsbad, CA). All chemicals used were of highest grade commercially available.

Cloning the Entire Open Reading Frame (ORF) of Sheep prnp Gene in Zero$^®$ Blunt Vector

Polymerase Chain Reaction (PCR) Amplification. The ovine prion gene was amplified by the polymerase chain reaction using the following oligonucleotide primers: forward 5'-TGCTGCAGACTTTAAGTGATT-3' and reverse 5'-CCCCAACCTGGCAAAG-3'. The PCR conditions were as follows; (i) Initial denaturation at 95 ºC for 1.5 minutes, (ii) denaturation at 94 ºC for 30 min, (iii) annealing at 58 ºC for 30 seconds and (iv) extension at 72 ºC for 30 seconds. Steps (ii) to (iv) were repeated 34 times. Since a native *Taq* polymerse enzyme was used for PCR, which does not have a proof-reading activity, it would add a 3' A-overhangs on both strands. To clean up the extra A's, the PCR product was incubated with 10 µL of 10µg/µL exonuclease-1 (product number 70073Z, USB, OH, USA) at 37 ºC for 30 minutes. The PCR product which was 893 bp was analyzed by 1.2% agarose gel electrophoresis for confirmation of the quality of PCR. The gel was analyzed by an imaging system (GDS 8000 system, UVP Bio-Imaging Systems, USA)

Cloning of PCR Product into TOPO-Vector. The purified DNA from the gel (PCR product) was cloned into the TOPO vector by Chemical Transformation using Invitrogen's Zero blunt PCR cloning kit (Carlsbad, CA) as described in manufacturer's instructions. Blue-white screening of the clones was done and the plasmid DNA was extracted from the positive clones as described in Sambrook and Russell (2001). Extracted DNA was used as a template for PCR to confirm the insert. The sequence of the isolated plasmid was confirmed by DNA sequence analysis with an Applied Biosystems Model ABI 3700 DNA analyzer.

Cloning the Coding Sequence for the Mature PrP^C Protein into pET102 Directional

TOPO Vector with a His -Tag

Expression Construct. The expression vector pET 102/D-TOPO was obtained

from Invitrogen Corporation, Carlsbad, CA (Cat # K102-01), which encodes a 13-kDa N-

terminal His-patch thioredoxin and has a C-terminal 6X-Histidine repeat which would

later help in the purification process. The 24 and 22 amino-acid signal peptide was

removed from N-and C-terminal respectively and primers were developed to amplify the

nucleotide sequence corresponding to the full-length mature PrP^C (25-234). PCR was

performed using the following oligonucleotide primers: forward 5'-CAC CAA GAA

GCG ACC AAA ACC TGG-3' and reverse 5'- CAC ACT TGC CCC CCT TTG GTA-

3'. The PCR was done using a high fidelity *taq* polymerase Platinum *pfx* DNA

polymerase and the PCR conditions were: (i) Initial denaturation at 95 ºC for 2 minutes,

(ii) denaturation at 95 ºC for 30 seconds, (iii) annealing at 58 ºC for 30 seconds, (iv)

extension at 72 ºC for 45 seconds and steps (ii) to (iv) were repeated 34 times.

Ligation and Transformation of pET 102/D-TOPO into One Shot TOP10

Competent Cells.   The ligation and transformation was done using the materials

obtained from Invitrogen's Champion pET Directional TOPO Expression kit (Cat #

K102-01) and by following the manufacturer's instructions. Positive clones containing

the complete plasmid were identified by plasmid isolation and PCR (using the above said

conditions). The sequence of the isolated PrP^C-6XHis gene was confirmed by DNA

sequence analysis (see above).

Overexpression of RecShPrP^C-6*His* into BL21 Star (DE3) One Shot Cells

The transformation reaction of 10 ml was added to two 250 ml of Luria-Bertani medium containing the antibiotic ampicillin (50 µg/ml) and was incubated at 37 ℃ with shaking at 250 rpms and was grown further until the OD_{600} reached between 0.5 – 0.8. These cultures were induced by the direct addition of 1mM IPTG (isopropyl-beta-D-thiogalactopyranoside) for 4 hours. The resulting cell pastes after centrifugation (3000g for 10 minutes at 4 ℃) were stored at -20 ℃.

Purification of the Fusion Protein RecShPrP Using Ni-NTA$^®$ Purification System

The purification was performed using the denaturing conditions as described in manufacturer's instruction Invitrogens' Champion TOPO$^®$ kit. The Guanidinium Lysis Buffer, pH 7.8 (supplied with the champion kit) was equilibrated to 37 ℃ and 0.2% sarkosyl salt was added to it. The harvested cell paste stored at -20 ℃ was thawed and resuspended in 8 ml of Guanidinium Lysis Buffer. The cells were rocked slowly at room temperature for 10 minutes to ensure thorough lysis and disrupted by sonication (three 3-second pulses at high intensity). The cell lysate was divided into 2 ml centrifuge tubes and was centrifuged at maximum speed for 1 minute at 4 ℃. The supernatant was aspirated into clean tubes. The pellets were spun again at maximum speed and the supernatant was aspirated and saved (10 µL) for SDS-PAGE analysis and further purification with Ni-NTA column. The aspirated supernatant was purified on a Ni-Purification Column (supplied with the kit).

The protein was allowed to bind with Ni^{2+}-nitrilotriacetate resin at room temperature for 30 minutes using gentle agitation to keep the resin suspended in the lysate solution. The resin was settled using centrifugation at 800 x g and the supernatant was aspirated carefully. The column was washed with 4 ml of Denaturing Binding Buffer by resuspending the resin and rocking for two minutes. The resin was settled by centrifugation as before and the supernatant was aspirated and saved at 4 ℃ for SDS-PAGE analysis. This step was repeated one more time.

The column was washed with 4 ml of Denaturing Wash Buffer (pH 6.0) by resuspending the resin and rocking for two minutes. The resin was settled by centrifugation as before and the supernatant was aspirated. The supernatant was saved at 4 ℃ for SDS-PAGE analysis.

The column was clamped in a vertical position and the cap on the lower end was snapped off. The protein was eluted by the addition of 5 ml of Denaturing Elution Buffer. Fractions of 1 ml were collected and the concentration was measured using a Nanodrop® ND-1000 Spectrophotometer (Delaware, USA) at the DNA-Protein Core Facility at Oklahoma State University, Stillwater (table).

All the fractions were concentrated by speed vacuuming using a SC 210A Savant Speed Vac System, Ramsey, MN at the DNA-Protein Core Facility at Oklahoma State University, Stillwater and stored at -20 ℃.

SDS and Western Blotting

Protein samples were analyzed by SDS/PAGE according to Laemmli (1970). Samples were denatured in 2x Laemmli Sample Buffer by boiling it for 5 minutes at 98

ºC and were loaded on a 15 % pre-cast Tris-glycine gel (Life gel Cat # NG 11-420) to do the electrophoresis (P8DS™ Emperor Penguin Vertical Electrophoresis System, Owl Separation System, Portsmouth, NH).   Protein staining of the gel was done with Coomassie Brilliant Blue. To perform western blotting, proteins from unstained SDS gel were transferred to a polyvinylidene diflouride (PVDF) membrane using HEP-1 Panther™ Semi Dry Electroblotting System (Owl Separating Systems, Portsmouth, NH) at 2.5mA/cm^2 of membrane surface area for 120 minutes. After blocking with 3% gelatin in 20mM Tris, 500mM NaCl, pH 7.5 (TBS), the membrane was incubated with primary antibody (anti-prion mAb raised in mouse) at room temperature for one hour. The membrane was washed thoroughly with 20mM Tris, 500mM NaCl, 0.05% Tween-20, pH 7.5 (TTBS) to remove any unbound antibody and incubated with secondary antibody (Goat anti-mouse IgG HRP conjugate from Bio-Rad Laboratories).   After thorough washing with TTBS, the labeled fusion protein was detected using the HRP color development reagent as the substrate containing 4-chloro-1-naphthol and hydrogen peroxidase.

The primary antibody used in this study was a monoclonal mouse anti prion IgG1 F89/169.1.5 (Sigma-Aldrich) that recognizes the amino-acid sequence IHFG of PrP^C and PrP^{Sc} from a variety of species including sheep.

Immuno-PCR

Working on ice, antigen was diluted in PBS- containing polypropylene tubes to the indicated levels, then 50 ml of each dilution were added to separate wells, which were left to bind antigen for 16 h at 4^oC. Coating antigen dilutions ranged from 200ng to

200fg/ml, decreasing in serial 100-fold dilutions. Unbound antigen was aspirated from

the wells, which were washed four times with 150 μl PBS containing 0.05% Tween-20.

Wells were blocked by incubation for 1 h at 37 ^{o}C with 150 ml of 10 mM Tris/HCl, pH

7.6, containing 5% w/v non-fat milk powder, 0.9% w/v NaCl, 0.05 mM Thimerosal (5%

milk powder buffer; MPB), then aspirated and washed as above. Primary antibody (50

μl) diluted as indicated in 0.5% MPB was added to wells and incubated for 2 h at 37 ^{o}C.

After washing as above, wells were further incubated for 1 h at 37 ^{o}C with 50 ml of

biotinylated donkey anti-mouse.immunoglobulin secondary antibody 2000-fold in 0.5%

MPB, then washed and incubated for 1 h at 37 ^{o}C with 50 ng Streptavidin in 50 ml of

0.5% MPB. Following washing to remove unbound Streptavidin, wells were incubated

with 854 fmol of biotinylated PCR target DNA in 50 ml of 0.5% MPB, for 1 h at 37 ^{o}C.

Unbound target DNA was then rigorously removed by washing wells five times with

PBSrTween, soaking wells for 3 min with wash buffer during each cycle, followed by

two washes with ultrapure, 0.2 mm filtered water. After addition of PCR reagents, well

contents were subjected to 30 cycles of PCR amplification, using Bio-Rad real time

thermocyler. PCR was performed in 15 μl mix containing 7.5 μl Syber Green mix (A&B

Warrington, UK), 0.3 μl each of forward and backward primer and 6.9 µL of autoclaved

ddH$_2$O.

RESULTS AND DISCUSSION

The conundrum surrounding the structural transformation of the normal prion

protein to the abnormal ones in the absence of any known chemical agent has intrigued

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the scientific community for a long period (Pan et al., 1993; Stahl et al., 1993). According to previous research, the abnormal isoform of prion acts as a template in the formation of nascent PrP^{Sc} from normal isoforms. However, more research needs to be done in order to understand their behavior in a multi-protein matrix and this can be achieved partly through the study of these two isoforms. Prions, as discussed earlier in the article (Kay, 2005) are a significant food safety concern. Research indicates that BSE in cattle is most likely caused by increased exposure of cattle to the scrapie agent, which survived in rendered sheep tissues, and ended up in meat and bonemeal (MBM; Taylor et al., 1997). Therefore, it is imperative to find out newer methods of detection and separation of the abnormal isoform of prions from the normal ones in both animal and human food systems. In order to learn more about these processes, it is important to study the two isoforms of prion at the molecular level. Molecular models of purified cellular PrP^{C} and PrP^{Sc} have been constructed using CD (secondary structure) and NMR (tertiary structure) techniques (Huang et al., 1994). However, because of the difficulties associated with obtaining large amounts of PrP^{C} from animal tissue, largely due to the highly unstable (Baron et al., 1999) and labile nature of PrP^{C}, (K. O'Rourke, USDA, ARS, Washington State University, Pullman, WA, personal communication) expression techniques have been developed to study the protein.

Initial attempts to express the whole protein using non-denaturing conditions resulted in low expression (Mehlhorn et al., 1996). Researchers subsequently attempted to express only the putative part of the secondary structure of the PrP^{C} residue (Gasset et al., 1992; Zhang et al., 1995; Mehlhorn et al., 1996). These experiments were more successful in large-scale expression and detection. Successful expression of the smaller

peptide residue has lead to further attempts to fully express the entire protein. Several expression systems were examined for large-scale production of PrP^C (Mehlhorn et al., 1996). Since initial attempts to express and purify parts of PrP^C were conducted using non-denaturing purification conditions, subsequent attempts looked at both non-denaturing and denaturing conditions. However, even under strong denaturing conditions, the efficiency and yield of purification was very low (Pergami et al., 1996; Turk et al., 1988; Scott et al., 1988; Caughey et al., 1988). The poor expression of the PrP^C during large-scale protein expression was largely accounted to the N-terminal basic amino acid positions at 22-29 of Syrian hamster PrP^C (Mehlhorn et al., 1996).

The normal prion-protein (PrP^C) and PrP^C-related proteins have been identified in many mammals, reptiles, birds and other organisms. However, only one study has been reported so far on the expression of recombinant sheep PrP^C ($RecShPrP^C$) at the protein level (Baron et al., 1999). In the current study, a Histidine tagged fusion system was used to overexpress the full-length (residues 25-234) of $ShPrP^C$ with *Escherichia coli* following the removal of the N- and C-terminal signal peptides, and experiments were conducted to detect $RecShPrP^C$ in a muscle protein mixture using Western blot and Immuno-PCR.

We chose to express sheep prion protein ($ShPrP^C$) for the large scale expression of the normal prion protein because $ShPrP^C$ is one of the most well defined and extensively studied prion proteins. Also from a biosafety standpoint, $ShPrP^C$ was safer to work with because its sequence differs substantially from humans implying a significant barrier for the transmission of sheep prions to humans (Prusiner, 1989). $ShPrP^C$ has shown to have an N-terminal signal peptide of 24 amino acids (Hope et al., 1986; Turk et

al., 1988). As a result, the entire ORF encoded by the *Ovis aries* (sheep) prion gene (*prnp*) was cloned because it would facilitate further cloning of the nucleotide sequence (coding exactly the full length mature protein from lysine at the 25 amino acid (aa) position to serine at 234 aa position).

Expression and Purification of the recombinant fusion protein *His*-PrP^C-(25-234) into pET102 Directional TOPO$^®$ Vector

The expression system pET102 Directional TOPO$^®$ Vector codes for a fusion protein of 366 amino acids, in which the sheep PrP^C sequence 25-234 was linked into a 42 amino acid C-terminal extension consisting a hexahistidine stretch which would facilitate the purification process by immobilized Ni-NTA chromatography column. The DNA sequencing of extracted plasmid-PrP^C gene verified its identity to sheep prion gene cDNA sequence by Heaton, M. P., Leymaster, K. A., Clawson, M. L. and Laegreid, W. W., (Animal Health Research Unit, U.S. Meat Animal Research Center, Box 166, State Spur 18D, Clay Center, NE, personal communication).

Determination of the molecular weight (Mw) of the prion protein with the fusion protein (His tag) by Expasy software (Swiss Institute of Bioinformatics, Geneva, showed it to be 40 kDa (39,581.09 daltons). The pI (Isoelectric point) of the protein was theoretically calculated as 7.77. According to Expasy, Mw of just the PrP (25-234) mature protein was calculated to be 22,815.77 daltons with a pI of 9.99.

In the past, research has been carried out to express the prion protein in part or as a full-length mature protein (amino acids 25- 234) in various bacterial systems. However, these experiments were not carried out under conditions identical to each other

(Mehlhorn et al., 1996). The expression was particularly low in the case of expressing the full-length mature protein either alone or as a fusion protein (Mehlhorn et al., 1996). Histidine tagged full-length mature part of RecShPrP^C (25-234) expressed in *E.coli* BL21 was solubilzed and extracted from inclusion bodies using 6 M guanidinium chloride. The cell extract was sonicated first and then loaded onto a Ni-NTA affinity column. His-PrP was eluted as a major fraction when the pH was changed from 7.8 to 4.0. The concentration of the protein was measured by the Nanodrop$^®$ ND-1000 Spectrophotometer (Delaware, USA) at the DNA-Protein Core Facility at Oklahoma State University, Stillwater and it was determined to be 0.96 mg/ml. Figure 1 (lane 3) and Figure 2 has the purified RecShPrP^C where a protein band of molecular weight 39 kDa can be seen. In lane 4 of Figure 1, the unpurified fraction is seen as a smear and the protein bands are not very clear. The recovery of recombinant protein from the inclusion bodies was maximized by the use of 6M guanidinium chloride, a low pH to guarantee protein stability, and a hexahistidine tag to permit purification by immobilized Ni(II) affinity chromatography. In our purified fraction, approximately 3% of the extracted protein was His-RecShPrP^C. Therefore, by keeping the protein constantly in an oxidized environment and maintaining the above said conditions, a yield of 28 mg of His-RecShPrP^C per liter of bacterial broth, which is slightly better than the yields recently reported for an amino-terminally polyhistidine-fused bovine prion protein (Negro et al., 1997) and scrapie protein (Baron et al, 1999). It might be possible to improve efficiency of the purification by recharging the Ni-NTA resin each time it is used. His-Select Nickel Affinity gel from Sigma-Aldrich, St. Louis, MO was selected because it gave

better results when compared to the affinity gel from Invitrogen Corporation, Carlsbad, CA

SDS-PAGE and Western Blot

The elution fraction obtained after purification of proteins induced from His-PrP transformed bacterial clones contained the detectable protein with a mobility of 39-kDa (expected size 39.5-kDa) as shown by the Coomassie blue staining of SDS-PAGE (Fig. 2, lane 2). The 39-kDa protein was recognized after Western Blotting by a monoclonal antibody (mouse IgG F89 / 160.1.5) against the prion protein (Fig. 3). Sequencing of the cloned insert showed the expected stop codon from the reverse primer (without stop codon) in frame with the prion sequence, which confirms the right fusion protein. The expressed recombinant fusion protein included the 23-kDa recombinant PrP (25-234), 13-kDa peptide on the N-terminal end of the plasmid, which corresponds to the His-patch thioredoxin, and a 3-kDa peptide on the C-terminal end containing the 42-amino acid V5 epitope and a hexahistidine (6xHis) region. The plasmid vector used for expression pET102/D-TOPO had a 126 amino acid sequence between the T7 promoter and PrP sequence, which might have reduced the efficiency of expression.

Immuno-PCR

Cervenakova and Brown (2004) reviewed several detection techniques of prion diseases and they commented immuno- PCR as a potential method for detecting PrP^{Sc} at very low concentrations. This technology, described previously by Case et al (1999) binds an antibody detection step similar to an ELISA with nucleic acid amplification by a real-time PCR procedure. Immuno-PCR results were analyzed by running the PCR

product on a 1.2% agarose gel electrophoresis and visualized by ethidium bromide staining and by observing the intensity and appearance of a band representing the right product size. Amplification was the highest for the 2 ng (lane 1) of RecShPrP^C protein followed by a decrease in the intensity at 200 femto-gram (lane 5) of prion protein (Figure. 4). Further enhancement of the assay specificity may be possible by the use of an automatic plate washer, which would give uniform and accurate reading of each wash process.

Our original plan was to extract prion protein directly from tissue in order to evaluate whether a post processing solubilization process on meat proteins destined for rendering or disposal could be used to extract the majority of the edible meat proteins (myofibrillar and sarcolasmic) away from prions in meat. However, as we had trouble like the other researchers in purifying prion protein, we started looking for alternative sources of large amount of PrP^C for biochemical studies. As a result, a protocol was developed to produce full-length mature sheep prion protein 25-234 (ShPrP [25-234]), fused with polyhistidine tag that can be easily cleaved after the purification step. The present expression system should help in producing large amount of normal prion protein thus overcoming the difficulties experienced in purifying sufficient amount the protein from meat solubilization studies. Future objectives include the use of a mammalian cell line for cloning the whole sheep *prnp* ORF without removing the signal peptides so that we can manifest the post-translational modifications present in prions from non-microbial sources. In addition, it is also suggested that the mammalian construct of RecShPrP^C subsequently can be utilized to conduct an in-vitro conversion of the PrP^C to PrP^{Sc} by

cyclic amplification of protein misfolding, resulting in indefinite amplification of PrP^{Sc} (Castilla et al., 2005).

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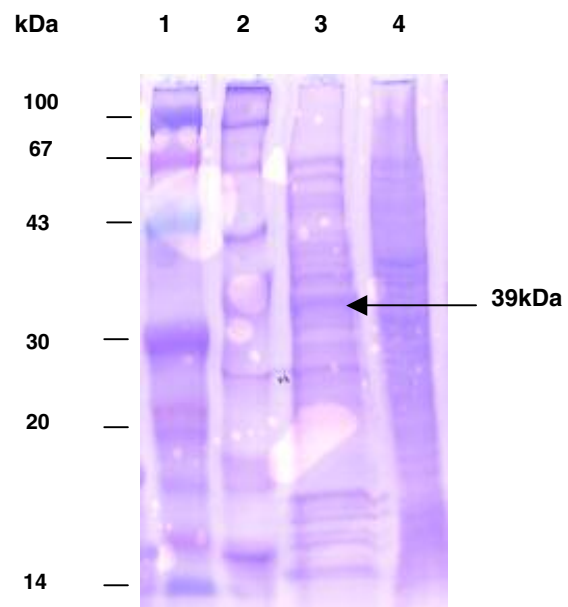
Figure 1

Figure 2

                        1   2

                                            ___ 39 KDa

Figure 3

600 bp →

1   2   3   4   5   6
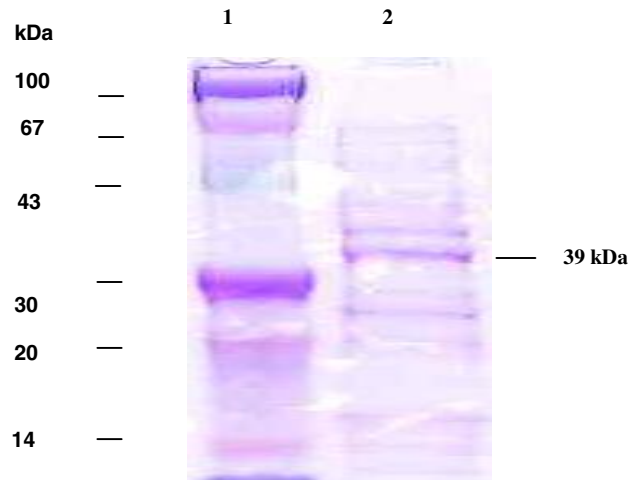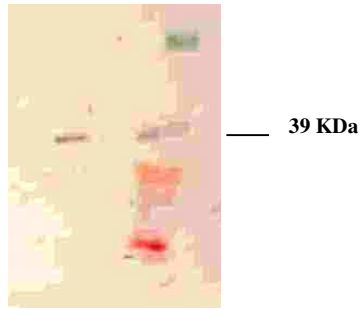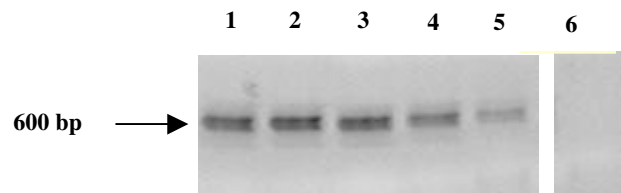
Figure 4

Figure Legends

Figure 1: Purified and Unpurified RecShPrP^C - Kaleidoscope ladder (Lane 1), Standard Biotinylated ladder (lane 2), purified PrP (10 μg) (lane 3) and unpurified PrP (lane 4).

Figure 2: SDS-PAGE analysis of the purified RecShPrP^C, Molecular Std (lane 1) and 1μg RecShPrP^C (lane 2)

Figure 3: Western Blot Analysis using the monoclonal antibody F89 / 160.1.5 following purification of RecShPrP^C. 1 μg of total protein was loaded in the lane 1 and 15 μg of kaleidoscope protein standard was loaded in lane 2. In lane 1, ~ 40 KDa is due to the RecShPrP^C.

Figure 4: RT-PCR products from an Immuno-PCR ran on a 1.2% agarose gel. Peptide was applied to wells in decreasing order of magnitude from 2 ng/ml to 200 fg/ml. Immuno-PCR products were analysed by 1.2% agarose gel electrophoresis and visualized by ethidium bromide staining. Each lane has prion protein ranging from 2 ng/ml to 200 fg/ml. 2 ng/ml (lane 1), 200 pg/ml (lane 2), 20 pg/ml (lane 3), 2 pg/ml (lane 4), 200 fg/ml (lane 5) and (Control) No target DNA (lane 6). The size of the PCR product was ~600 base pairs.

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APPENDIX 1

MATERIALS AND METHODS

Preparation of Muscle Protein and its Combination with Recombinant Prion Protein (RecPrP)

Experiment 1. Experiments 1 and 2 were performed in order to extract myofibrillar protein from meat and combine it with the extracted sheep RecPrP^C. However due to technical errors, we ended up extracting more of sarcoplasmic and very less myofibrillar protein.

Comminuted lean beef muscle was diluted (1:9) with cold (4 ^{o}C) ddH2O and homogenized at 15,000 rpm using a polytron (Kinematica GmbH, Lucerne, Switzerland) for 30 seconds (Dewitt et al., 2002). Samples were then centrifuged at 10,000g for 10 minutes at 4 ^{o}C. The supernatant was collected and analyzed using a Nanodrop$^®$ ND-1000 Spectrophotometer (Delaware, USA) located at the core facility of Oklahoma State University, Stillwater. Purified recombinant sheep prion protein (RecShPrP^C) was added to the supernatant to a final concentration of 1% and 10%. SDS-PAGE electrophoresis was performed as described by Laemmli (1970) with following modifications *(39)*. Equal amounts of 2X Laemmli sample buffer was combined with protein- RecShPrP^C mixture and was boiled for 5 minutes. 20, 15 10 and 2 μg of RecShPrP^C protein-meat protein complex was loaded on a 15% pre-cast Tris-Glycine gel (Bio-Rad Laboratories, Hercules, CA, Cat # 1611103) and run for 2 hours at 120 volts. Western Blot was performed following the protocol mentioned previously.

Experiment 2. Purified RecShPrP^C was combined directly to the comminuted lean beef muscle to a final concentration of 10% and was diluted with cold (4 ^{o}C) ddH$_2$O

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(1:9) and homogenized at 15,000 rpm using a polytron for 30 seconds. The sample was centrifuged at 3000g at 4 $^{\circ}$C for 10 minutes. The supernatant was collected and stored at 4 $^{\circ}$C for doing SDS-PAGE.

SDS-PAGE electrophoresis was performed as described by Laemmli (1970) with following modifications. To prevent the proteins from precipitation at low pH, samples were diluted 1:4 with 5% SDS and 200 µL of the SDS-diluted sample (30 and 20 µg of RecShPrP^{C}) was added to 50 µL of 1X Laemmli sample buffer and boiled as previously stated before loading.

RESULTS AND DISCUSSION

The concentration of extracted sarcoplasmic protein at OD_{280} was measured to be 15 µg/µL. In experiment 1, the mixture of recombinant fusion prion protein and muscle protein contained two detectable proteins with mobilities of 39 and 43-kDa, as shown by the Commassie blue staining of SDS-PAGE (data not shown). However, western blot using antibodies against the prion protein did not give any signal (data not shown). The most likely explanation for the negative results might be that both RecShPrP^{C} and the sarcoplasmic protein got degraded in the presence of naturally occurring proteases. A second possible explanation for a negative Western Blot result might be inefficient transfer of protein from the gel to the PVDF membrane. Another possibility is that our detection system, which was a colorimetric assay, was less sensitive towards the color reaction when compared to other detection assays like chemiluminiscence assay.

The results for Experiment 2 also yielded negative and did not give any signal on the western blot analysis.

APPENDIX 2

Preparation of 1.2% agarose gel

1. Weigh 1.2 g of agarose (Cat # Sigma St. Louis MO) and add it to 100 mL TBE buffer in an Erlenmeyer flask.

2. Heat it in a microwave (approximately for 2 minutes) so that the agarose melts and being careful, that it does not boil and spill all over.

3. Working under the hood, add 7 µL of ethidium bromide (Cat # 7627, Sigma St. Louis MO) to the melted agarose while it is still hot and mix it by gently swirling it under the hood. (Note: Ethidium bromide is a carcinogen)

4. Make the gel cast (Cat # Bio-Rad) ready by taping both its ends to contain the melted agarose and then pour the agarose into it. Place the comb and let it set.

10X TBE Buffer

Tris Base (Cat # T4661 Sigma St. Louis MO): 108g

Boric acid: 55g

EDTA (Cat # E7889 Sigma St. Louis MO): 9.3g

ddH_2O to: 1L

Total Volume: 1L

APPENDIX 3

Extraction of Genomic DNA from sheep tissue

1. Weigh out approximately 1 gram of sheep tissue (in our case we used obex region sheep brain, however for DNA extraction, any tissue like blood can be used) in a 50 mL centrifuge tube and homogenize it with in 3mL of ice-cold lysis buffer (10 mM Tris-Cl, pH 8.0, 0.1 M EDTA (pH 8.0), 0.5% (w/v) SDS, 20 µg/ml DNase-free pancreatic RNase) using a tissue homogenizer (Tissumizer, Tekmar Model Number SDT 1810 OH,USA).

2. To the cell lysate, add 50µL of proteinase K (200 µg/ml) (Cat # 82452 Sigma St. Louis MO) which would digest any protein and thus would increase the yield of genomic DNA.

3. Incubate the digest at 60ºC in a water bath or an incubator for 3 hours and transfer the lysate to a new 50 mL centrifuge tube.

4. Extract DNA with 5 mL of phenol: chloroform: isoamyl alcohol (25:24:1) by spinning the tubes at 14000 rpm (Beckman J2 HS, USA) for 5 minutes at 4 ºC and transfer the supernatant to a new centrifuge tube.

5. Precipitated the DNA in the aqueous phase with 1 volume of 100% ethanol and 1mL 10M ammonium acetate.

6. Determine the concentration of extracted genomic DNA using a Nanodrop® ND-1000 Spectrophotometer (Delaware, USA). The concentration of DNA measured in this project was 1.178 mg/µL.

7. Dilute the DNA to final working concentration of 100 ng/mL and store at -20 ºC.

8. Add 6 µL of DNA (100 ng/mL) to 2 µL of 6X sample buffer and load it on a 1.2%

    agarose gel. Separate the DNA in an electric field by running at a voltage of 140 V

    for 20 minutes. This step is done in order to see if the extracted DNA is denatured

    or not.

9. Analyze the gel using an imaging system (GDS 8000 system, UVP Bio-Imaging

    Systems, USA) to check the quality of obtained DNA.

6X Sample Buffer

Bromophenol blue: 2.5 ml

Xylene cyanol: 2.5 ml

Sucrose: 4.0 ml

ddH$_2$O to   10 ml

Total Volume   10 ml

APPENDIX 4

Primer Design

1.   This protocol is for developing primers for the nucleotide sequence encompassing the complete coding sequence the prion gene that is approximately 771 bases.

2.   Copy the complete coding sequence of the prion gene in the FASTA format from the NCBI site (http://www.ncbi.nlm.nih.gov) to the Sequence Input Box on the Primer3 site (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

3.   Using the Primer3 website, pick a primer set (left and right) and optimize the various parameters (length of the primers, self-complementarity of the sequence, GC content, Tm difference etc) for an efficient primer giving minimal misbinding.

4.   Analyze the designed primers using IDT's Oligo Analyzer 3.0 (http://207.32.43.70/biotools/oligocalc/oligocalc.asp) for various parameters to ensure maximum efficiency.

5.   Once the primer is developed, get it ordered from Integrated DNA Technologies Inc (or any other vendor).

6.   Working on ice, touch spin the obtained primers briefly on a table top centrifuge (5415 C Eppendorf, Westbury, NY) to collect all the primer, which is in the powder form to the center of the vial before diluting. Dilute the primers to make 200 μM of stock solution by using the following equation. The molecular weight of the forward primer used in this part of the current project was 6451.2 mg

Volume of ddH$_2$O needed in μLs =

The amount of ddH$_2$O added to forward primer =   186 μL.

7. Similarly calculate the amount of ddH$_2$O needed for the reverse primer. The final working concentrations required for both primers are 25 µM which can be achieved by doing the following calculation.

$V_1 C_1 = V_2 C_2$, where

V_1 = Volume of the first solution

C_1 = Concentration of the first solution

V_2 = Volume of the second solution

C_2 = Concentration of the second solution

APPENDIX 5

Polymerase Chain Reaction (PCR)

1.   Make the following recipe for the PCR mix:

DD H_2O:	14.5 µL
PCR buffer (10X)	
(Cat # E08663, Applied Biosystems, Foster City, CA):	2 µL
$MgCl_2$ (25mM)	
(Cat # E08688, Applied Biosystems, Foster City, CA):	1.2 µL
Forward and Reverse primers (20mM):	0.4 µL
dNTPs (Cat # 170-8874, Bio-Rad Laboratories, Hercules, CA):	0.4 µL
Taq polymerase:	0.25 µL
Genomic DNA (100ng):	1.0 µL

Total	20.0 µL

2.   Touch spin the PCR mix on a table top centrifuge (5415 C Eppendorf, Westbury, NY) and put it on a thermocycler (DNA Engine DYAD®, Bio-Rad Laboratories Inc, South San Francisco, CA) with the following PCR conditions:

(i)   Initial denaturation at 95 ºC for 3 minutes,

(ii)   Denaturation at 94 ºC for 30 seconds,

(iii)   Annealing at a 58 ºC for 30 seconds (This can be achieved by doing a

          gradient first).

(iv)   Extension at 72 ºC for 2 minutes and repeat steps (ii) to (iv) 34 times.

3.   After the completion of PCR, add 2 µL of 6X sample buffer to the PCR product and

     run it on a 1.2% agarose gel (with ethidium bromide) to see the PCR results.

4   Visualize the gel using an imaging system (GDS 8000 system, UVP Bio-Imaging

     Systems, USA) to analyze the PCR results.
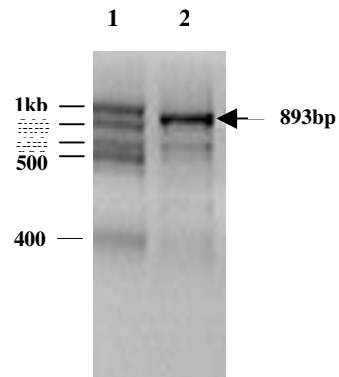
Agarose gel (1.2%) analysis of PCR product amplified using the extracted genomic DNA as the template for PCR. 1kb standard marker (lane 1) and 893 bp PCR product amplified off the genomic DNA (lane 2).

APPENDIX 7
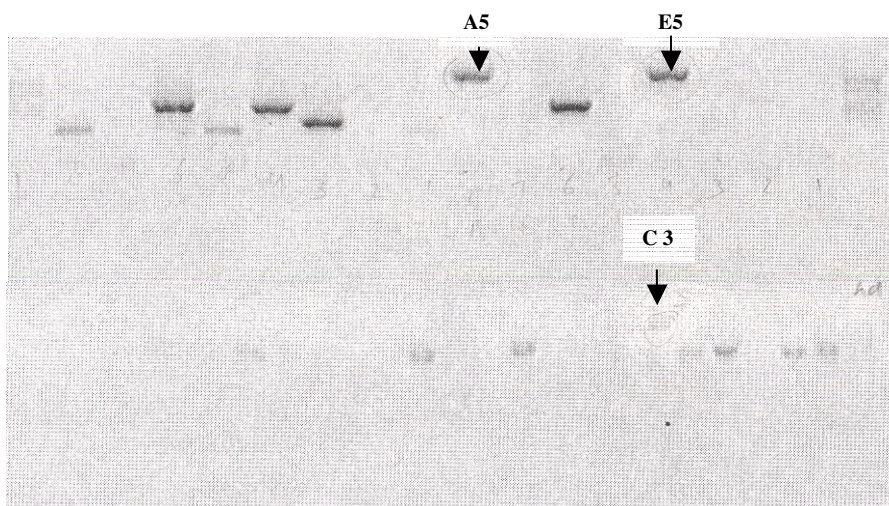
Agarose gel analysis of the PCR amplified product of 893 bases using the DNA from cell burst as the template for PCR. Upon screening for clones which has taken the insert using PCR, three positives A5, E5 and C3 were detected which had the DNA band of the right size of 893 base pairs.
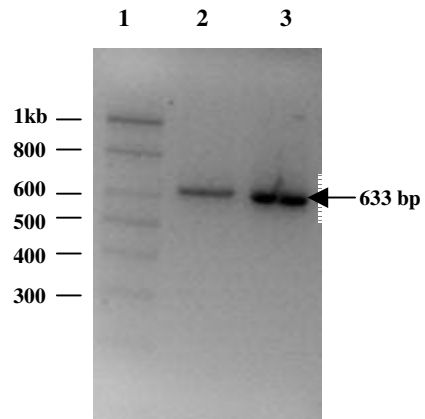
PCR product with and without stop codon - 1kb standard marker (lane 1), PCR products amplified off the ORF of PrP with stop codon (lane 2) and without stop codon (lane 3). Note that both the products have the same size of 633 bp, which encodes the 210 amino acids (GenBank accession DQ345757).
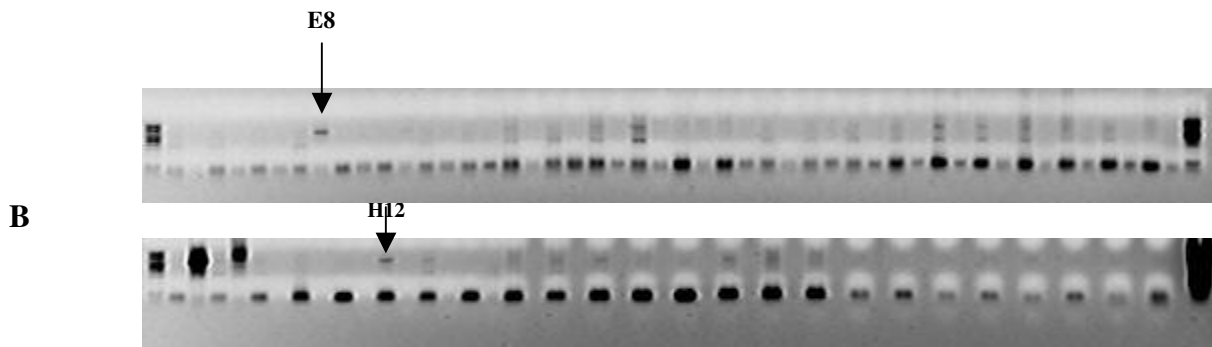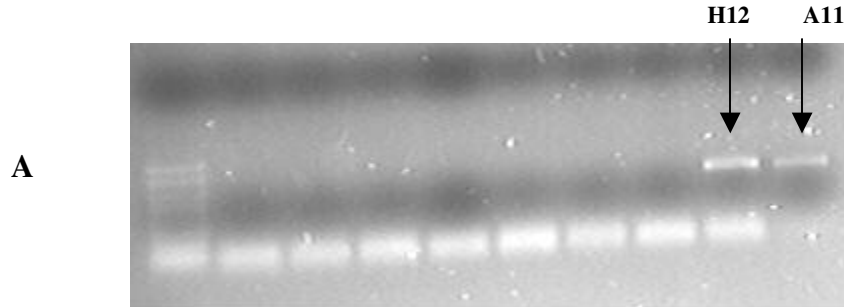
Agarose gel analysis of PCR product, which is the coding DNA sequence corresponding to the mature prion protein PrP-(25-234), Screening of Positives Clones With and Without Stop Codons - Out of more than 50 clones screened, only 4 clones were positive for the right insert of 633 base pairs. **Figure A**. H12 and A11 were the positive clones with the stop codons, which we did not use it for expression. **Figure B**. E8 and H12 were positive with out a stop codon. E8 was used for the expression purposes.

DNA sequence analysis of the complete ORF of the prion protein gene (*prnp*) of clone C3

GANTG AATAC GACT CICTATAGGGCGCGAATT GGGCCCTCTAG NTGCAT GCTCGAGCGGCCGCCGCCNGTGTGATNNNTATNTGCAC NATTCAGGCCCCAAC CTGGCAAAGAGATTAAGAA GATAAT GAAAACAGGAAGGTTGCC

10   20   30   40   50   60   70   80   90   100   110   120   130

CCTATCCTACTATGAGAAAAATGAGGAAGAGATGAGGAGGATCACAGGAGGGGGAAGAAAAGAGGACCACACTTGCCCCCTTGGTAATAAGCCTGGGATTCTCTCTGGTACTGGGGTGATGCACA

140   150   160   170   180   190   200   210   220   230   240   250   260

TTTGCTCCACCACTCGCTCCATTATCTTGATGTCAGTTTCGGTGAAGTTCTCCCCCTTGGTGGTGGTGACTGTGTGTTGCTTGACTGTGATGTTGACACAGTCATGCACAAAGTTGTTC

270   280   290   300   310   320   330   340   350   360   370   380

TGGTTACTATACTGATCCACTGGTCTGTAGTACACTTGGTTGGGGTAACGGTACATGTTTTTCACGATAGTAACGGTCCTCATAGTCATTGCCAAAATGTATAAGAGGGCCCTGCTCATGGCACT

390   400   410   420   430   440   450   460   470   480   490   500

DNA sequence analysis of the complete ORF of the prion protein gene (*prnp*) of clone C3

TCCCAGCATGTAGCCACCAAGGCCCCCTACCACTGCTCCAGCTGCAGCAGCTCCTGCCACATGCTTCATGTTGGTTTTTTGGCTTACTGGGCTTGTTCCACTGACTGTGGCTACCACCTTGAC

10   520   530   540   550   560   570   580   590   600   610   620   630

CCCAGCCTCCACCACCATGTGGCTGTCCCAGCCACCACCATGGGGGCTGACCCCAGCCACCTCCATGAGGTTGGCCCCAGCCACCTCCATGGGGGCTGACCCCAGCCACCCCCTCCCTGAGGT

640   650   660   670   680   690   700   710   720   730   740   750

DNA sequence analysis of the complete ORF of the prion protein gene (*prnp*) of clone A5

PRISM
Basecaller-POP6.bcp   #6-universal
BC 1.1.0.0   Lane 39
Points 3396 to 19798   Base 1 : 3396
Thu, Aug 25, 2005 5:43 PM
Spacing: 11.77{11.77

GNTTTATAC GACTGCTATAGGGCGAATT GGGCCCTCT AGATGCAT GCT C GAGC GGC C CCC NGTGT GA TNNNA TNT GCAC NATTCA GGC CCCAAC CT GGCAA AGATTAAGAA GATAAT GA AAACA G GAAGGTT GCC
10   20   30   40   50   60   70   80   90   100   110   120   130

CC TATCC TACTA TGA GAAAAT GA GGAAA GA GA TGA GGA GGA TCACA GGA GGGGGAAGAA GAGGAT CACAGCTT GCC CCCC TTT GGTAA TAAGCCT GGGATTCTCT CT CGG TACT GGGT GA T GCACA T
140   150   160   170   180   190   200   210   220   230   240   250   260

TTT GC TCCACCACTC GCT CCATTAT CTTGAT GTCAG TTTCGGT GAAGTTCTCCCC CTT GGT GGT GGT GACT GTG TGTT GCTT GACT GT GATGTT GACACAGTCAT GCACAAA GTTGTTCTG
270   280   290   300   310   320   330   340   350   360   370   380

GTTACTATACT GATCCACTGGTCTGTAGTACACTTGGTT GG GGTAACGGGTACATGTTTTCACGATAGTAACGGTCCTCATAGTCATTGCCAAAATGTATAAGAGGCCCTGCTCATGGCACTTC
390   400   410   420   430   440   450   460   470   480   490   500   510

DNA sequence analysis of the complete ORF of the prion protein gene (*prnp*) of clone A5

CCAGCATGTAGCCACCAAGGCCCCTACCACTGCTCCAGCTGCAGCAGCTCCTGCCACATGCTTCATGTTGGTTTTTTGGCTTACTGGGCTTGTTCCACTGACTGTGGCTACCACCTTGACCCC
520   530   540   550   560   570   580   590   600   610   620   630

AGCCTCCACCACCATGTGGCTGTCCCCAGCCACCACCATGGGGCTGACCCCAGCCACCTCCATGAGGTTGGCCCCCAGCCACCTCCATGGGGCTGACCCCAGCCACCCCCTCCCTGAGGTGGATAGG
640   650   660   670   680   690   700   710   720   730   740   750   76

:GGTTGCCTCCAG
50   770

DNA sequence analysis of the complete ORF of the prion protein gene (*prnp*) of clone E5

GNTTTTATACGACTCTATAGGGCGAATTGGGCCCTCTAGATGCATGCTCGAGCGGCCGCCNGTGTGAANTTM TNTNCNCNATTCAGGCCCCAACCTGGCAAAGATTAAGAATGAAAAACAGGAAGGTT
10   20   30   40   50   60   70   80   90   100   110   120   130

ECCCTATCCTACTATGAGAAAAATGAGGAAAGAGATGAGGAGGATCACAGGAGGGGGAAGAAAAGAGGATCACACTTGCCCCCCTTTGGTAATAAGCCTGGGATTCTCTCTGGTACTGGGTGATGCA
140   150   160   170   180   190   200   210   220   230   240   250   260

CATTTGCTCCACCACTCGCTCCATTATCTTGATGTCAGTTTCGGTGAAGTTCTCCCCTTGGTGGTGGTGACTGTGTGTTGCTTGACTGTGATGTTGACACAGTCATGCACAAAGTTGTT
270   280   290   300   310   320   330   340   350   360   370   380

CTGGTTACTACTATGATCCACTGGTCTGTAGTACACTTGGTTGGGGTAACGGGTACATGTTTTTCACGATAGTAGTAACGGTCCTCATAGTCATTGCCAAAATGTATAAGAGGCCTGCTCATGGCACT
390   400   410   420   430   440   450   460   470   480   490   500

Letter of Permission for the use of Prion Protein Image

Thomas, Stanley

From:	Sarit Helman [shelman@cmpharm.ucsf.edu]	**Sent:**	Wed 5/17/2006 6:23 PM
To:	Thomas, Stanley		
Cc:			
Subject:	prion protein image		
Attachments:	📄 Educational PrP image.tiff(9MB)		

Dear Stanley,

Please use the attached image of normal and abnormal prion protein in your thesis. It is more up-to-date than the one you had sent me. We do not charge for educational use.

If, however, you want to use our image in a for profit publication, we will ask that you purchase one. When you are ready for that, please contact me again and I will send you the appropriate information.

Good luck with your thesis!

--
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VITA

STANLEY THOMAS

Candidate for the Degree of

Master of Science

Thesis : Production of Recombinant Sheep Prion Protein (RecShPrP^C) and its Detection in Muscle Foods Using Western Blotting and Immuno-PCR

Major Field : Food Science

Biographical

Personal Data: Born in Allepey, Kerala, India, July 16, 1977, the son of M. K. Thomas and Leela Thomas

Education : Graduated from Kendriya Vidyalaya (Central School), Bolarum, Hyderabad, India, May 1995; Received Bachelor in Science in Hotel Management and Catering Technology from Osmania University.

Experience : Employed by FrontlineSoft Limited, Hyderabad, India as a Team Leader in the Sales Department 2001-2002, Hotel Grand Kakatiya Sheraton, Hyderabad, India, as Production Intern in 1999, Kodak India Limited, Hyderabad, India, as a Marketing Intern in 2001, Oklahoma State University, Animal Science Department as a Lab Assistant and a Research Assistant 2003-present.

Professional Memberships: Institute of Food Technologists, American Meat Science Association.

Name: Stanley Thomas   **Date of Degree**: July, 2006

Institution: Oklahoma State University   **Location**: Stillwater, Oklahoma

Title of Study: PRODUCTION OF RECOMBINANT SHEEP PRION PROTEIN (RecShPrP^C) AND ITS DETECTION IN MUSCLE FOOD USING WESTERN BLOTTING AND IMMUNO-PCR

Pages in Study: 81   **Candidate for the Degree of Master of Science**
Major Field: Food Science

Scope and Method of Study: The objectives of this study were to over-express the sheep prion protein (RecShPrP^C) using a bacterial vector. The prion protein gene was PCR amplified using sheep genomic DNA and was cloned into pET102/D-TOPO plasmid. The integrity of the cloned PCR product as well as the *His*-tagged fusion protein and its orientation within the vector was determined by DNA sequencing and upon confirmation was induced with isopropyl-beta-D-thiogalactopyranoside for protein expression. The expressed proteins were solubilized by addition of 6M guanidinium chloride in lysis buffer and purified using adsorption to a Nickel-Nitriloacetic^{++} metal affinity resin. Production of RecShPrP^C protein by this method could be used to study of solubilzation and fractionation of prion proteins from muscle proteins. Separation of prion proteins from the myofibrillar and sarcoplasmic proteins may enhance the safety of meat products that would eventually be rendered and used for non-mammalian animal feed or fertilizer.

Findings and Conclusions: We developed a protocol to produce full-length mature sheep prion protein 25-234 (ShPrP [25-234]), fused with polyhistidine tag that can be easily cleaved after the purification step. The present expression system should help in producing large amount of normal prion protein thus overcoming the difficulties experienced in purifying sufficient amount of the protein for solubility studies.

ADVISER'S APPROVAL: ___Christina M. DeWitt_____